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Direct Single-Reagent Fluorescence Polarisation Immunoassay for Valproic Acid in Serum

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Summary: A fluorescence polarisation immunoassay for quantitating serum concentrations of valproic acid was developed and validated. Its low molecular weight and lack of structural features caused difficulties in producing suitable antibodies. However, success was achieved using 2-propyl-6-aminohexanoic acid to make the fluorescein-labelled drug and two immunogens, the first using glutaraldehyde to link the drug derivative to keyhole limpet haemocyanin, and the second by carbodiimide activation of cellulose hydroxyl groups and coupling them to the drug derivative and killed *Mycobacteria*. It was found that both immunogens produced a good antibody response in sheep. The antibodies were highly specific and the assay results correlated well with an in-house gas-liquid chromatographic method.

Introduction

During recent years, valproic acid has become a first-choice drug in the treatment of most types of epilepsy. There is increasing interest in its quantitative measurement in plasma or serum for the detection of poor compliance and because of the great individual differences in dosage requirements to achieve therapeutic concentrations (1, 2). Various assay methods have been introduced including gas-liquid chromatography (3), high-performance liquid chromatography (4) and immunoassays (5). Enzymeimmunoassays and fluorescence polarisation immunoassays are now the most widely used in therapeutic drug monitoring due to their simplicity, and the availability of automated or semiautomated instruments and commercial kits (6). In addition to their speed and high sample throughput these methods are reliable and specific and the results obtained correlate well with chromatographic methods (7).

It proved difficult to raise suitable antibodies against valproic acid because of its small size and structural simplicity. Coupling the drug directly to keyhole limpet haemocyanin through its free carboxyl group, or coupling dipropyl malonic acid (2-carboxy valproic acid) to the carrier protein proved unsuccessful; both resulted in the production of ovine antibodies able to bind the fluorescein-labelled derivative prepared through the same functional group, but not the unlabelled drug. However, a suitable antibody response is produced in sheep immunised with an immunogen prepared by coupling the hydrocarbon chain of the molecule, via a suitable functional group, to keyhole limpet haemocyanin, and the resulting antisera used with a fluorescein-labelled derivative prepared through the same route. These reagents were employed to develop the present single-reagent fluorescence polarisation immunoassay (FPIA) for the direct determination of valproic acid in serum.

Materials and Methods

Reagents

Sodium valproate was a gift from Reckitt and Colman, Kingston-upon-Hull, U. K. Valproate metabolites were gifts from Dr. *H. Schafer*, Desitin-Werk Carl Klinke GmbH Hamburg, F. R. G. and from Sanofi UK Ltd, Manchester, U. K. Fluorescein isothiocyanate isomer 1 (FITC), Sigmacell type 20, and most chemicals used in synthesis and cross-reactivity experiments were obtained from Sigma, Poole. Dorset. U. K.: keyhole

limpet haemocyanin from Calbiochem-Behring, San Diego, CA 92112, U.S.A.; silica gel thin-layer chromatography (TLC) plates (type PLK5F) from Whatman, Maidstone, Kent, U.K.; triethylamine from Koch-Light, Colnbrook, Bucks, U.K.; Triton X-100 surfactant, sodium azide, sodium lauryl sulphate and all organic solvents ("Analar" grade) from BDH Chemicals, Poole, Dorset, U.K.; and pooled normal human serum from ILS, London EC1, U.K. Other drugs employed in the specificity studies were donated by their respective U.K. distributors.

Assay standards

A 1 g/l solution of valproic acid in methanol was prepared and added to drug-free pooled normal human serum to obtain the following concentrations: 10, 20, 50, 100, and 200 mg/l.

Patients' specimens

Serum samples from patients receiving valproic acid, with or without other antiepileptic drugs, were obtained and assayed by a gas-liquid chromatographic method (8).

Diluent buffer

Sodium phosphate buffer (100 mmol/l, pH 7.5) containing per litre, 1.0 mg sodium lauryl sulphate, 2.2 mg Triton X-100 and 1 g of sodium azide as an antibacterial agent.

Assay tubes

Disposable 50 × 10 mm round glass cuvettes (no. 9518), from Abbott Diagnostic Division, Basingstoke, Hampshire, U.K.

Polarisation fluorometer

A Model 4000 polarisation fluorometer (SLM Instruments, Urbana, IL 61801) was employed, as described previously (8), except that the sample compartment was fitted with an adaptor to accept the cylindrical cuvettes.

Preparation procedures

2-Propyl-6-aminohexanoic acid

Sodium hydride (0.43 g, 10.7 mmol) was dissolved in 30 ml of dry dimethyl formamide and reacted with 2 ml (9.7 mmol) of diethylpropylmalonate. After 30 min with continuous stirring, 2.7 g (9.7 mmol) of N-(4-bromobutyl) phthalimide was added and refluxed for 1.5 h. The reaction mixture was diluted with ether, washed several times with water, dried over magnesium sulphate and the solvent evaporated under reduced pressure to yield a yellow oil. This was purified by flash column chromatography (petroleum ether/diethyl ether, 2 + 1 by vol) to obtain 3.6 g (91% yield) of a white solid diethyl-(4-N-butyl phthalimide)propyl malonate. This diester (0.85 g, 2.1 mmol) and 0.5 ml (10 mmol) of hydrazine were then dissolved in 30 ml methanol and refluxed for 4 h. After removing the solvent, the residue was dissolved in 1 mol/l HCl, washed with chloroform, made alkaline with 2 mol/l NaOH and extracted with chloroform. The organic phase was washed with water, dried over magnesium sulphate and the solvent evaporated under vacuum to yield an oil which was dissolved in 15 ml of 6 mol/l HCl and refluxed overnight. The reaction mixture was neutralised, the water evaporated and the residue extracted with methanol. Upon filtration and removal of the solvent, 0.23 g of a white solid (2-propyl-6-aminohexanoic acid) was obtained (fig. 1).

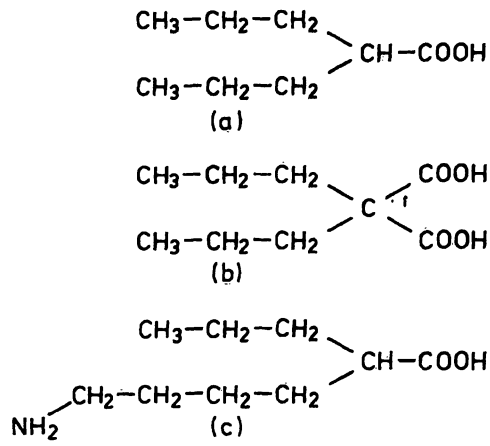


Fig. 1. Structural formulae of (a) valproic acid, (b) dipropyl malonic acid, and (c) 2-propyl-6-amino hexanoic acid.

Valproate immunogen I

2-Propyl-6-aminohexanoic acid (50 mg, 0.3 mmol) was reacted with 1 ml of 50 g/l glutaraldehyde (0.6 mmol) in 1 ml of 1 mol/l acetate buffer pH 7.0. After 5 min of continuous mixing, this was added to a solution of 60 mg keyhole limpet haemocyanin in 5 ml acetate buffer and stirred for 30 min. Then a solution of 100 mg sodium borohydride in 2 ml distilled water was added; the reaction mixture stirred for 15 min; transferred to a dialysis bag and dialysed for 3 days against three changes of 5 litres of phosphate buffered saline (50 mmol/l, pH 7.4). When lyophilised, it yielded 76 mg of the conjugate.

Valproate immunogen II

Carbonylimidazole (30 mg) was added to a suspension of Sigmacell Type 20 cellulose in 2 ml of dry acetone and stirred for 1 h at room temperature. Then a solution of 2-propyl-6-aminohexanoic acid (50 mg, 0.3 mmol) and triethylamine (50 µl) in 1 ml of acetone was added. After stirring for 1 h, 30 mg of a suspension of killed *Mycobacteria* in water was added. The reaction mixture was stirred overnight, dialysed as above and lyophilised to yield 146 mg of the conjugate.

Fluorescein tracer

Ten mg (0.06 mmol) of 2-propyl-6-aminohexanoic acid and 10 mg (0.03 mmol) of fluorescein isothiocyanate isomer-1 were dissolved in 2 ml methanol containing 1 ml/l of triethylamine. After stirring for 2 h, in the dark, at ambient temperature, the solution was acidified with 1 mol/l HCl and then filtered. The precipitate obtained was dissolved in methanol and purified by thin-layer chromatography, using methylene chloride/methanol (1 + 1 by vol) to separate a major band (R_f 0.3). The band was scraped from the plate, extracted with methanol and stored at −20 °C. The concentration of the fluorescein tracer was estimated spectrophotometrically as described previously (10).

Anti-valproic acid anti-sera

Three ewes were immunised with immunogen I (no. 101, 102, and 103) and an additional 3 ewes with immunogen II (no. 14, 15, 16) according to a protocol described elsewhere (10).

Procedures

Gas-liquid chromatography procedures

A Pye Unicam series 304 instrument linked to a Hewlett Packard 3380A integrator was used for routine determination of valproic acid in serum samples following an established method (8). Briefly, each serum sample was acidified with 1 mol/l HCl and added to a glass tube containing benzyl alcohol as internal standard. Then the sample was extracted into 1 ml chloroform of which 5 µl was injected directly onto a column packed with 100 g/l diethylglycol succinate on Chromosorb W. H. P. 100/120 mesh.

Fluorescence polarisation immunoassay (FPIA) procedures

Premixed reagent: A solution of fluorescein labelled valproate in assay buffer was prepared at a concentration of 30 nmol/l. To this was added anti-valproate anti-serum (pre-diluted 100-fold) in the ratio 2:1. The reagent was then stored at 4 °C in the dark until required.

Assay protocol: Assays were performed at room temperature in duplicate. To 25 µl of diluted serum standard or sample (pre-diluted 50-fold in diluent buffer) was added 1.5 ml of the premixed reagent in the glass cuvette. The cuvettes were vortex mixed, incubated for 10 min before fluorescence polarisation was measured.

Results

Characterisation of the valproate immunogens

Immunogens I and II were hydrolysed by refluxing with 10 ml of dilute HCl for 1 h. Upon diluting the product in the assay buffer and measuring the concentration of valproate by the present assay, a ratio of "valproic acid equivalent" to the carrier was obtained: 0.7 mmol/g (2300:1 in molar terms) for immunogen I and 0.03 mmol/g for immunogen II.

Anti-valproate anti-sera

All sheep produced antibodies that bound valproic acid but the antisera varied significantly in their titres. The immunoglobulin fraction of each serum and of a non-immunised sheep serum were obtained by conventional sodium sulphate precipitation (11) and doubly diluted in diluent buffer. Antibody dilution curves were constructed by adding 1 ml aliquots of these dilutions to 0.5 ml of the fluorescein tracer (30 nmol/l), measuring fluorescence polarisation and making a background correction. Figure 2 shows such curves for the most responsive sheep to each immunogen (no. 102 and 14) and for a non-immunised sheep. The antiserum from sheep 102 was used in all further studies.

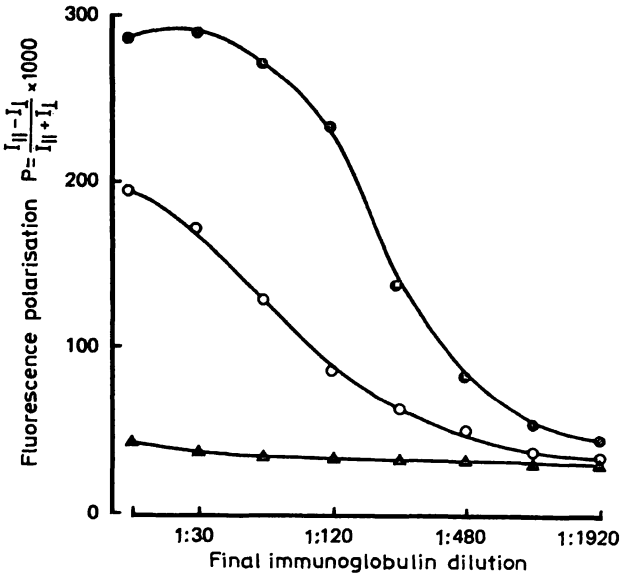


Fig. 2. Dilution curves of the immunoglobulin fractions of anti-valproic acid antisera obtained from
the second bleed of sheep 102 (●),
the eighth bleed of sheep G14 (○) and
a non-immunised sheep serum (▲).
The polarisation of fluorescence (P) is defined as:

$$P = \frac{I_{II} - I_{\perp}}{I_{II} - I_{\perp}},$$

where
I_{II} = intensity of the fluorescence when the exciting light is vertically polarised (parallel to the direction of the electric vector of the exciting wave)
$I_{\perp}$ = intensity of the fluorescence when the exciting light is horizontally polarised (perpendicular to the direction of the electric vector of the exciting wave)
As a quotient, P has the unit 1 (one, dimension-less).

Optimisation of assay conditions

The presence of albumin and other serum proteins increased the fluorescence polarisation signal due to non-specific binding with the fluorescein tracer. This was abolished by incorporating 1.0 mg/l of sodium lauryl sulphate as a blocking agent in assay diluent buffer. Triton X-100 was added in equimolar concentration (2.2 mg/l) in the assay buffer to overcome the antigen-antibody inhibition effect caused by sodium lauryl sulphate (12).

Assay kinetics

Association kinetics

The association of the fluorescein tracer with the valproate antibodies was investigated by obtaining serial polarisation readings after adding 0.5 ml of tracer (30 nmol/l) to 1 ml of the antiserum (diluted 100-fold), which were plotted against time on linear paper. The polarisation readings increased rapidly and reached a plateau within 45 s.

Dissociation kinetics

The rate of dissociation was investigated as previously described (6). Two populations of antibody were found, one with a polarisation half life of about 57 s, and the other, a faster dissociating antibody, with a half-life of about 24 s. Both constitute a substantial proportion of the total antibody populations.

Incubation time

Each standard was assayed and polarisation readings taken at timed intervals. The assay reached equilibrium after 10 min and the polarisation readings were stable for at least 45 min.

Standard curve

A typical standard curve (fig. 3) was obtained using 25 µl of buffer or serum standard (prediluted 50-fold) which covered the clinically relevant range for valproate.

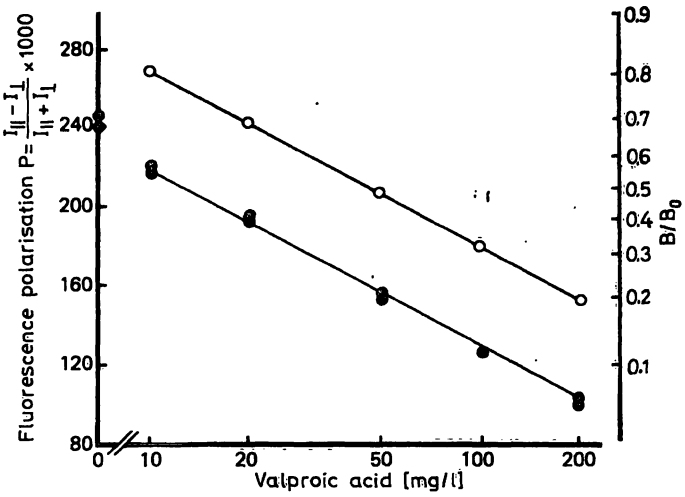
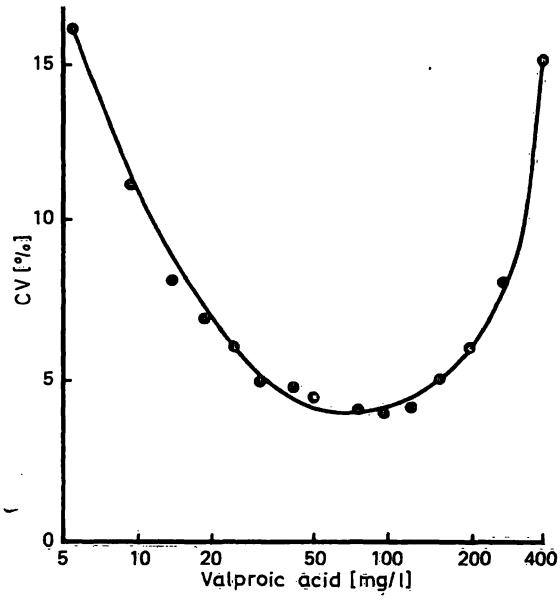


Fig. 3. Standard curves for valproic acid obtained by fluorescence polarisation (●) and from the Logit-Log transformation (○).
For further explanations see fig. 2.



Assay validation

Sensitivity

The sensitivity of the assay was determined by the *Rodbard* method (13). The minimal detectable concentration of valproic acid was 1.9 mg/l and the minimal detectable dose was 0.95 ng per tube.

Imprecision

Three selected samples of patients' sera were each assayed ten times in one assay, and gave mean results of 37, 90, and 132 mg valproic acid per litre with CVs of 3.7%, 3.5% and 3.8% respectively. The same samples were assayed on ten separate occasions and gave between-assay CVs of 5.9%, 4.1%, and 3.1% respectively. A precision profile was calculated using the Edinburgh immunoassay program (14) from the data collected from a standard curve and the simultaneous assay of 50 patients' samples. Within the boundaries of the standard curve the CVs were all below 10% (fig. 4).

Analytical recovery

Valproic acid was added at concentrations of 10, 20 and 50 mg/l to four patients' samples containing valproic acid in concentrations ranging from 0 to 125 mg/l. Average analytical recovery was 99.6%, 97.2% and 95.0% respectively.

Linearity and parallelism

The standard curve for valproic acid exhibited good linearity (fig. 3). When a patient's serum containing a high concentration of valproic acid (130 mg/l) was diluted 2-, 4-, 8-, and 16-fold in diluent buffer, the values obtained were linear and parallel to the normal standard curve.

The results obtained from a typical standard curve were processed by the Logit-Log program (15) converting polarisation readings to B/B_0 values. A straight line was obtained which was parallel to the normal standard curve (fig. 3).

Specificity

Antibody specificity

To assess the specificity of the anti-valproate antibodies, the following chemicals, sharing some structural similarity to valproic acid, were tested for their cross-reactivity at a concentration of 1 g/l; ethanoic acid, propanoic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, norvaline, valine, amino-*n*-valeric acid, ornithine, valeric acid ethyl ester and benzoyl amino-*n*-valeric acid. The aliphatic carboxylic acids did cross-react, but not significantly — all less than 2%. All other analytes showed zero cross-reactivity, demonstrating the high specificity of the antibodies produced.

Clinical specificity

The cross-reactivity of the main metabolites of valproate (16) were determined at a concentration of 1 g/l, relative to valproic acid. Those which cross-reacted significantly at this level were further assessed by *Abraham*'s method (17). The results are shown in table 1. None cross-reacted to an extent likely to cause inaccuracy in the assay of patients' samples. Other antiepileptic drugs and their major metabolites showed no cross-reaction when tested at concentrations up to 1 g/l.

Tab. 1. Cross-reactivity of valproic acid metabolites

Compound	% Cross-reactivity by *Abraham*'s method	% Cross-reactivity at 1 g/l
(1) 2-propyl-2-pentenoic acid (2-en valproic acid)	18.3	15.0
(2) 2-propyl-4-pentenoic acid (4-en valproic acid)	36.7	25.0
(3) 3-hydroxy-2-propyl pentanoic acid (3-OH valproic acid)	—	<1.0
(4) 4-hydroxy-2-propyl pentanoic acid (4-OH valproic acid)	—	1.0—2.0
(5) 5-hydroxy-2-propyl pentanoic acid (5-OH valproic acid)	22.2	20.0
(6) 3-oxo 2-propyl pentanoic acid (3-keto valproic acid)	—	<1.0
(7) 2-propyl-4-methyl butyrolactone (4-hydroxyvalproic acid lactone)	—	1.0
(8) 2-propyl pentyrolactone (5-hydroxyvalproic acid lactone)	—	1.0—2.0
(9) 2-*n*-propyl glutaric acid	—	<1.0

Accelerated stability study

A bulk (50 ml) of the premixed reagent was prepared and after overnight incubation, a standard curve was obtained. The reagent was then stored in the dark at 37 °C for a week, after which a comparable standard curve was obtained, indicating that the single reagent should be stable for a year at 4 °C (18).

Correlation with gas-liquid chromatography method

Results of the assay of 46 patients' serum samples by this assay (y) and by the chromatographic technique (x) were related by the regression line $y = 1.0x + 2.9$ ($r = 0.99$) which was calculated assuming that the two methods have equal precision characteristics (19).

Discussion

We describe another "single-reagent FPIA" utilizing the short dissociation half-lives of most hapten : antibody complexes (6, 18). For such assays to be feasible, one requires antiserum which contains a substantial population of fast dissociating antibodies. In spite of the initial difficulties encountered in raising antibodies to such a small molecule, we were eventually successful in producing an antiserum which contains a substantial proportion of fast dissociating antibodies. These antibodies consist of two major populations with polarisation half-lives of 24 s and 57 s, which have allowed us to develop the present single-reagent FPIA for valproic acid.

The additional advantage of such assays is the long-term stability of the premixed reagent as shown by the accelerated stability study. The premixed reagent was stable and gave comparable results when stored at 37 °C for 1 week which implies one year's stability at 4 °C (18).

In raising antisera to valproic acid two immunogens were prepared by coupling 2-propyl-6-aminohexanoic acid to two different carrier molecules. The high valproate/keyhole limpet haemocyanin incorporation ratio in immunogen I may have resulted in the greater antibody response in the immunised sheep, as com-

pared with the second immunogen (valproate/Sigmacell-*Mycobacteria* conjugate).

The specificity studies show that highly specific antibodies were produced, with, as expected, optimum selectivity for the carboxylic acid group of valproic acid. The introduction of a hydroxyl or a oxo group close to the carboxylic acid grouping prevents antibody binding, but a hydroxyl group further along the molecule is less effective and 5-hydroxy valproic acid therefore shows greater cross-reactivity than its 4-hydroxy and 3-hydroxy counterparts. The presence of an additional carboxyl group in 2-*n*-propyl glutaric acid, although at the far end of the molecule, prevented antibody binding presumably by modifying the polarity, electron density, and the effective charges of the molecule, thereby interferring with the forces involved in antigen-antibody binding.

The only other cross-reacting compounds were 2-en and 4-en valproic acid suggesting that some antibody populations cannot distinguish between unsaturated and saturated compounds. The 4-en compound is the more immunoreactive probably because of the relative positions of the carbon-carbon double bonds. However these cross-reactants will not interfere with the assay of patients' samples since the plasma concentrations of all the metabolites are considerably lower than those of the parent drug (16). This is shown by the correlation coefficient (r) of 0.99 obtained when the results of the immunoassay are compared with those of the gas-chromatography method.

All other reliability criteria of the assay are satisfactory and the small volume (0.5 µl) required provides another advantage when limited sample volumes are available especially in paediatric practice.

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